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Reactive oxygen species production and redox state in parthenogenetic and sperm-mediated bovine oocyte activation

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Abstract

The knowledge concerning redox and reactive oxygen species (ROS)-mediated regulation of early embryo development is scarce and remains controversial. The aim of this work was to determine ROS production and redox state during early *in vitro* embryo development in sperm-mediated and parthenogenetic activation of bovine oocytes. Sperm-mediated oocyte activation was carried out in IVF-modified synthetic oviductal fluid (mSOF) with frozen–thawed semen. Parthenogenetic activation was performed in TALP plus ionomycin and then in IVF-mSOF with 6-dimethylaminopurine plus cytochalasin B. Embryos were cultured in IVF-mSOF. ROS and redox state were determined at each 2-h interval (7–24 h from activation) by 2′,7′-dichlorodihydrofluorescein diacetate and RedoxSensor Red CC-1 fluorochromes respectively. ROS levels and redox state differed between activated and non-activated oocytes ($P<0.05$ by ANOVA). In sperm-activated oocytes, an increase was observed between 15 and 19 h ($P<0.05$). Conversely, in parthenogenetically activated oocytes, we observed a decrease at 9 h ($P<0.05$). In sperm-activated oocytes, ROS fluctuated throughout the 24 h, presenting peaks around 7, 19, and 24 h ($P<0.05$), while in parthenogenetic activation, peaks were detected at 7, 11, and 17 h ($P<0.05$). In the present work, we found clear distinctive metabolic patterns between normal and parthenogenetic zygotes. Oxidative activity and ROS production are an integral part of bovine zygote behavior, and defining a temporal pattern of change may be linked with developmental competence.

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Introduction

Oxidative stress has been widely reported in biological sciences to describe an enhanced state of oxidants in cells, a situation in which the concentration of reactive oxygen species (ROS) increases above its biologically normal levels (Sikka 2001). Oxidative stress, mediated by ROS, results in an imbalance of the intracellular redox potential toward an oxidized potential (Balaban *et al.* 2005). The role of ROS in biological processes is still controversial. It was found that the oxidative modification of cell components due to the action of ROS is one of the most potentially damaging processes for normal cell function, leading to inactivation of proteins, lipid membrane peroxidation, and DNA alterations (Yang *et al.* 1998). However, it has been observed that at physiological concentrations, ROS participate in normal cell processes as major factors in growth and development regulation (Hancock *et al.* 2001).

The procedure for producing embryos *in vitro* in cattle is still unsatisfactory, with results ranging from 35 to 50% blastocyst rate at day 7/8 of development (Lim *et al.* 2007, Shirazi *et al.* 2009). Chemical activation presented significantly lower success rates and blastocyst formation compared with IVF (Ruggeri *et al.* 2012), demonstrating that the process of oocyte activation is a major factor for successful production of reconstructed embryos by somatic cell nuclear transfer (Wells *et al.* 1999). ROS generation has been implicated as a major cause of poor development of bovine embryos *in vitro*. ROS have been suggested to participate in meiotic arrest in oocytes (Nakamura *et al.* 2002) and embryonic block and cell death (Hashimoto *et al.* 2000). It has been proposed that high levels of ROS may cause oocyte meiotic arrest (Downs & Mastropolo 1994). Within the oocyte, a critical intracellular concentration of ascorbic acid is necessary for normal cytoplasmic maturation and embryo developmental competence (Tatemoto *et al.* 2001). It has been observed that an excessive amount of glucose in the maturation medium produces high ROS concentrations and exerts a negative effect on subsequent bovine embryo development to the blastocyst stage (Hashimoto *et al.* 2000). The importance of regulating ROS levels is revealed by the observation that cumulus–oocyte complexes (COCs) have developed significant antioxidant strategies to control ROS

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production (Cetica *et al*. 2001, Tatemoto *et al*. 2001, Dalvit *et al*. 2005*a*).

On the other hand, some evidence exists demonstrating that ROS are important to spermatozoa in regulating every aspect of sperm function examined, including their movement characteristics, capacitation, sperm–zona interaction, acrosome reaction, and sperm–oocyte fusion (Baker & Aitken 2004, Rivlin *et al*. 2004). Some studies have also documented that the addition of natural antioxidants to oocyte maturation medium failed to modify the percentage of bovine embryos produced *in vitro* (Blondin *et al*. 1997) or even diminished the rate of embryo production (Dalvit *et al*. 2005*b*). Other cell-permeable antioxidants inhibited the precocious resumption of meiosis in rat oocytes, suggesting a regulatory function of ROS in the maturation process (Takami *et al*. 1999).

Several transcription factors involved in diverse developmental processes are now known to be regulated by the intracellular redox potential (Imai *et al*. 2000, Dickinson & Forman 2002, Zhang *et al*. 2002, Rahman *et al*. 2004, Liu *et al*. 2005, Funato *et al*. 2006). The recent discovery that these factors can be sensitive to oxidation by ROS or *S*-glutathionylation or require NAD(P)H (the reduced form) or $NAD(P)^+$ (the oxidized form) is generating new insights into the regulation of embryonic development (Dumollard *et al*. 2007). It has been observed that redox state and ROS levels are negatively associated within the cell. A high cellular oxidative activity (e.g. increased mitochondrial oxygen consumption rate) is usually associated with lower ROS production and *vice versa* (Boveris & Cadenas 1982). In the early mouse embryo, the fundamental importance of redox state and ROS regulation of early embryo development has also been demonstrated (Dumollard *et al*. 2007).

Previous studies from our group have documented variations in ROS production attributable to oocyte and early embryo metabolic activities during bovine *in vitro* maturation (IVM) and embryo development (Dalvit *et al*. 2005*a*, Morado *et al*. 2009). In addition, temporal changes in oxygen consumption were detected in bovine oocytes undergoing the transition from oocyte to zygote (Lopes *et al*. 2010). Accordingly, the aim of this work was to determine the production of ROS and redox state during early *in vitro* embryo development in sperm-mediated and parthenogenetic activation of bovine oocytes.

Results

Sperm-mediated oocyte activation

In sperm-mediated activation, following insemination, pronuclei formation began by 9 h and peaked at 13–15 h (around 80% of the zygotes), and then slightly decreased until 24 h. Syngamy began around 13 h and reached a plateau at 17–24 h (around 40% of the zygotes). First cleavage of embryos began at 21 h (Fig. 1).

Parthenogenetic oocyte activation

In parthenogenetically activated oocytes, by the first observation at 7 h, all oocytes were at the pronuclear stage and remained at this stage up to 17 h from the initiation of activation, when they abruptly decreased. Chromosomal fusion and cleavage began at 17 h, with most two-cell parthenotes (around 76%) appearing about 19–21 h (Fig. 2).

Redox state in non-activated, sperm-activated, and parthenogenetically activated oocytes

In sperm-activated oocytes, oxidative activity presented an increase between 15 and 19 h ($P<0.05$; Figs 1 and

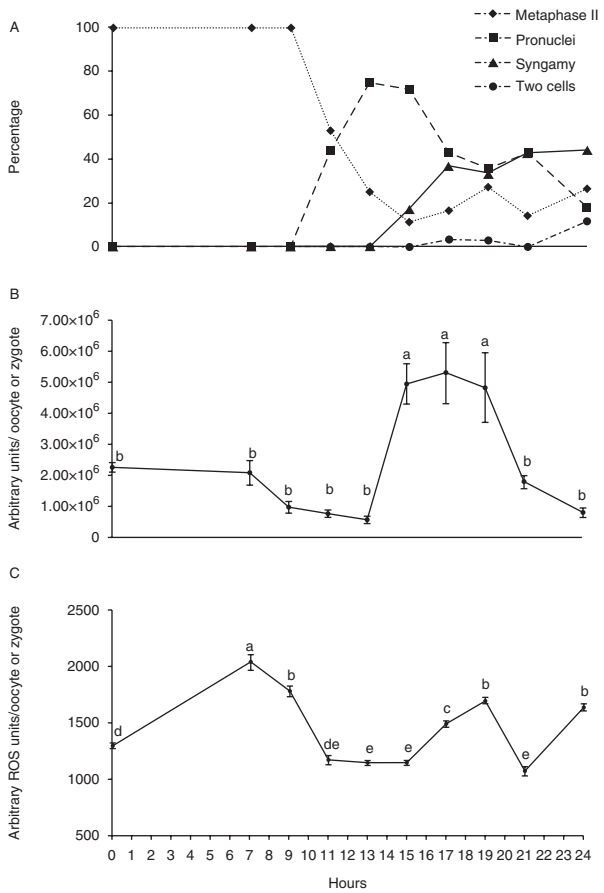

Figure 1 (A) Nuclear stage of putative zygotes in sperm-mediated oocyte activation, $n=1612$ zygotes. (B) Redox state in sperm-mediated oocyte activation. Values are expressed as mean arbitrary units/oocyte or zygote ± S.E.M., $n=134$ putative zygotes. ^{a,b}Values with different superscripts are significantly different ($P<0.05$). (C) Reactive oxygen species production/total esterase activity in sperm-mediated oocyte activation. Values are the ratio between DCHFDA and FDA assays; they are expressed as mean arbitrary ROS units/oocyte or zygote ± S.E.M., $n=1478$ putative zygotes. ^{a,b,c,d,e}Values with different superscripts are significantly different ($P<0.05$).

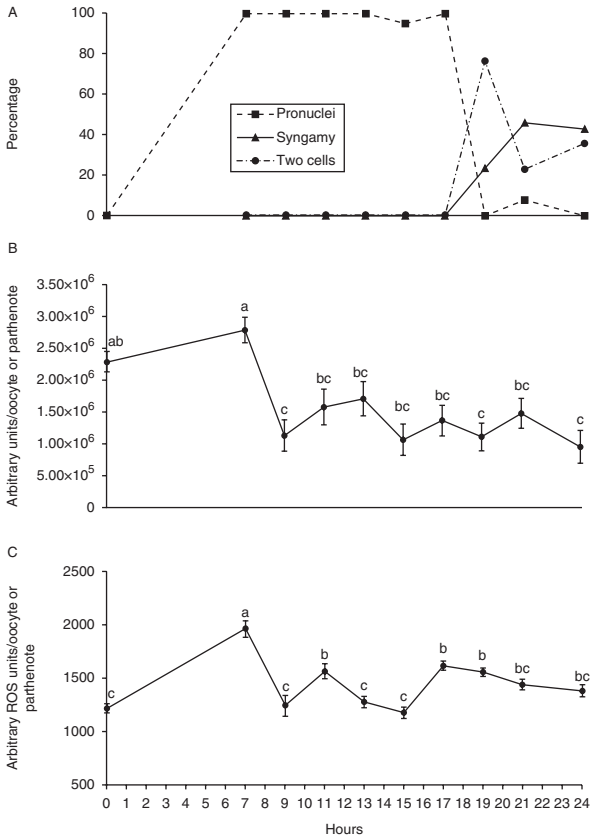

Figure 2 (A) Nuclear stages observed in parthenogenetic oocyte activation, $n=630$ parthenotes. (B) Redox state in parthenogenetically activated oocytes. Values are expressed as mean arbitrary units/oocyte or parthenote ± S.E.M., $n=172$ parthenotes. [a,b,c]Values with different superscripts are significantly different ($P<0.05$). (C) Reactive oxygen species production/total esterase activity in parthenogenetically activated oocytes. Values are the ratio between DCHFDA and FDA assays; they are expressed as mean arbitrary ROS units/oocyte or parthenote ± S.E.M., $n=458$ parthenotes. [a,b,c]Values with different superscripts are significantly different ($P<0.05$).

3a, b, c, and d). On the other hand, in parthenogenetically activated oocytes, we observed a decrease at 9 h ($P<0.05$), and no further alteration until 24 h (Figs 2 and 3e, f, g, and h).

To determine whether the oxidative activity detected during early oocyte activation depended on activation or simply reflected the length of time since maturation, IVM non-activated oocytes were cultured *in vitro* for 24 h, revealing a significant decrease between 11 and 17 h ($P<0.05$; Fig. 4).

ROS production in non-activated and sperm-activated and parthenogenetically activated oocytes

ROS production was compared between sperm-activated oocytes and parthenogenetically activated oocytes. In sperm-activated oocytes, ROS levels fluctuated throughout the 24 h of development, presenting clearly discernable peaks around 7, 19, and 24 h ($P<0.05$; Figs 1 and 5a, b, c and d), while in parthenogenetically activated oocytes, peaks were detected at 7, 11, and 17 h ($P<0.05$; Figs 2 and 5e, f, g and h).

To determine whether the rise in ROS levels depended on oocyte activation or reflected ROS production in the aging matured oocyte, non-activated oocytes were cultured *in vitro* for 24 h, in which we observed a significant decrease after 0 h ($P<0.05$; Fig. 4).

In the experiment carried out to demonstrate the specificity of the dyes, we discovered that oocytes incubated with pyruvate presented a significant decrease in ROS production compared with the control (pyruvate, 606.27 ± 30.97 vs control, 989.14 ± 43.24 arbitrary ROS units/oocyte; $P<0.05$; $n=30$ oocytes for each group). On the other hand, no difference in the oxidative activity was observed between the two treatments (pyruvate, $(3.962\pm0.216)\times10^6$ vs control, $(4.323\pm0.117)\times10^6$ arbitrary units/oocyte; $n=30$ oocytes for each group).

Discussion

To our knowledge, this is the first time significant shifts in both ROS production and redox state have been observed in association with temporal developmental events in bovine oocyte sperm-mediated and parthenogenetic activation. Different temporal patterns of nuclear

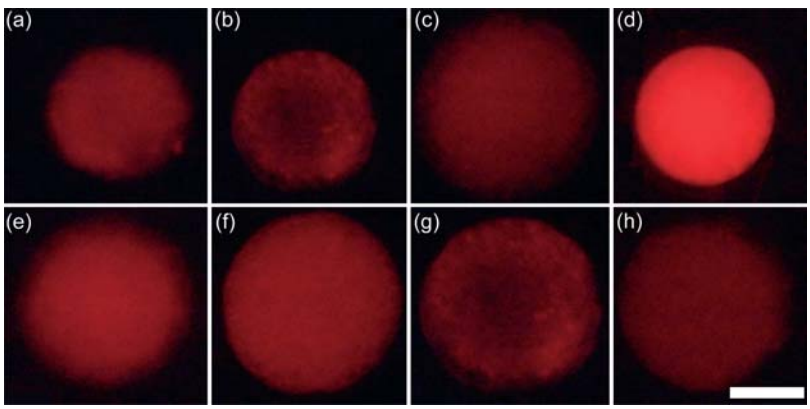

Figure 3 Representative activated oocytes or putative zygotes stained with RedoxSensor Red CC1 (×120). (a, b, c, and d) Sperm-activated oocytes at 0, 7, 11, and 19 h from activation and (e, f, g, and h) parthenogenetically activated oocytes at 0, 7, 11, and 19 h from activation. Bar = 50 μm.

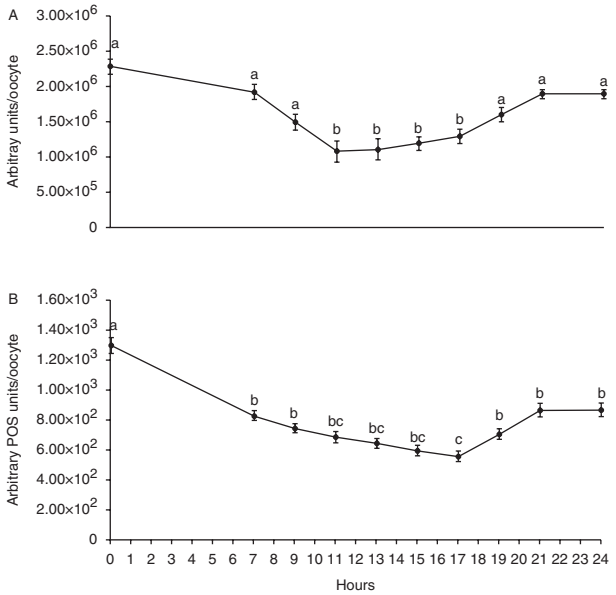

Figure 4 (A) Redox state in non-activated oocytes. Values are expressed as mean arbitrary units/oocyte±S.E.M., $n=180$ oocytes. [a,b]Values with different superscripts are significantly different ($P<0.05$). (B) Reactive oxygen species production/total esterase activity in non-activated oocytes. Values are the ratio between DCHFDA and FDA assays; they are expressed as mean arbitrary ROS units/oocyte±S.E.M., $n=200$ oocytes. [a,b,c]Values with different superscripts are significantly different ($P<0.05$).

events were observed between the two types of activation. Within putative zygotes stemming from sperm-mediated activation, there was a temporal spread of the major developmental events after fertilization. In contrast, and not surprisingly, parthenogenetically activated oocytes behaved in a highly synchronized manner throughout development to the first cleavage division. The temporal sequence of developmental events observed with the two types of activation are similar to those previously reported for bovine zygotes (Gordon 1994). The difference in the patterns observed is attributed to the asynchrony of sperm penetration, which lasts about 4 h in bovines (Jiang 1991). Nevertheless, the synchronous nuclear progression observed in parthenogenetic activation does not necessarily equate to improved embryo development *in vitro* (Monaghan 1993).

RedoxSensor Red CC-1 is a fluorescent dye that has been used as an indicator of oxidative activity in living cells (Chen & Gee 2000). The increase in oxidative activity observed in sperm-activated oocytes corresponds to the initiation of pronuclear formation and first mitotic division in putative zygotes, suggesting increased demands for energy for these events. It has been observed that one- and two-cell bovine embryos are dependent on mitochondrial oxidative phosphorylation for energy supply, consuming oxidative substrates to produce ATP (Kim *et al*. 1993, Thompson *et al*. 1996). Coincidently, a higher oxygen consumption rate was detected before cleavage in bovine zygotes (Lopes *et al*. 2010).

In contrast, parthenotes initially have a high oxidative activity, which then declines from 7 h following activation and remains low thereafter during the developmental process with some small, non-significant, oscillations despite the events of chromosomal fusion and first cleavage. To our knowledge, there is no published data concerning the metabolism of bovine parthenotes, but an increase in the metabolic activity is expected in any type of embryo that undergoes cell division. In coincidence with our findings, in mouse, parthenogenetic one- to two-cell embryos present a lower glucose metabolism, glycogen content, ATP content, and adenylate kinase activity than fertilized embryos (Han *et al*. 2008). This difference in metabolic behavior between the two groups of activated oocytes could in part be responsible for the markedly lower developmental competence of the parthenogenetically activated oocytes.

It has been shown that the DCFHDA probe is oxidized by hydrogen peroxide, its derived oxidants, other peroxides, and indirectly by the superoxide anion when generating hydrogen peroxide, thus providing a useful test to evaluate ROS production (LeBel *et al*. 1992). In sperm-activated oocytes, ROS peaks appear before and/or during structural events associated with early embryo cleavage. The first peak occurs during

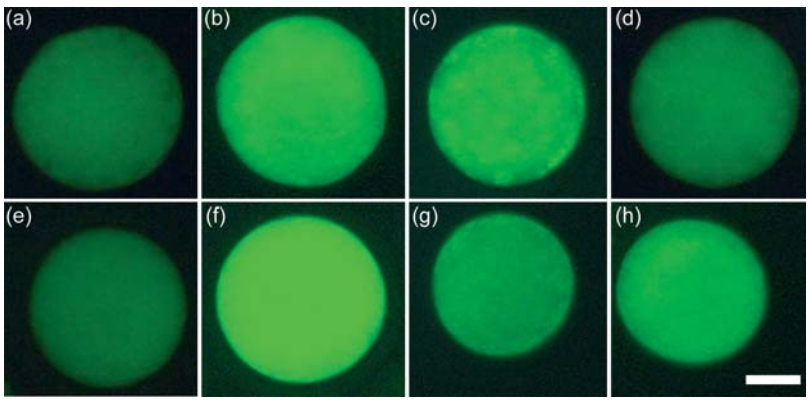

Figure 5 Representative activated oocytes or putative zygotes stained with 2′,7′-dichlorodihydrofluorescein diacetate (×120). (a, b, c, and d) Sperm-activated oocytes at 0, 7, 11, and 19 h from activation and (e, f, g, and h) parthenogenetically activated oocytes at 0, 7, 11, and 19 h from activation. Bar=50 μm.

preparative stages before pronuclear formation (7 h), such as sperm penetration and sperm head decondensation and the second and third peaks with association of pronuclei (19 h) and first mitotic division (24 h) respectively. In contrast, in non-activated oocytes, ROS levels dropped after 7 h culture and remained low until 24 h. These results are in agreement with those reported for murine zygotes, in which only fertilized oocytes showed a rise in ROS production, while unfertilized oocytes presented declining levels over the same period (Nasr-Esfahani & Johnson 1991). It has been suggested that certain levels of ROS are needed for the interaction between the spermatozoa and oocytes during bovine IVF, indicating that they may play different roles depending on the moment and the quantity in which they are present (Blondin *et al*. 1997). Very recently, a new class of dioxygenases has been identified, the Ten-eleven translocation proteins (Tet 1–3) that are key to the hydroxylation of 5-methylcytosine to 5-hydroxymethylcytosine, thereby initiating the first steps toward DNA demethylation (Kriaucionis & Heintz 2009, Tahiliani *et al*. 2009). As DNA demethylation is a key process within early embryo development immediately following fertilization and pronuclear formation, perhaps the rise we have observed here in ROS production and elsewhere in oxygen consumption (Lopes *et al*. 2010) reflects Tet protein activity. In support of this, bovine pronuclear zygotes have significant levels of hydroxymethylcytosine in the male pronucleus, but not the female pronucleus, which appears to be largely due to the activity of Tet 3 (Wossidlo *et al*. 2011).

In parthenotes, high levels of ROS were observed, coinciding with a high rate of oocytes at the pronuclear stage (7 and 11 h) and before cleavage (17 h). Once again, ROS production seems to be associated with structural events related to early embryo development. Coincidently, an increase in ROS production in parthenogenetically activated murine oocytes was also observed (Nasr-Esfahani & Johnson 1991).

Of interest was the lack of synchronicity between the peaks in ROS levels and those of oxidative activity in both types of activation; indeed, their behavior was contrasting during development. It is known in somatic eukaryotic cells that mitochondria in a resting respiratory state (state 4) produce higher levels of ROS than those with active oxygen consumption (state 3); some of the electrons passing through the mitochondrial electron transport chain are transferred to molecular oxygen to form superoxide anion, which can then derive hydrogen peroxide (Boveris & Cadenas 1975, 1982).

During fertilization, the stimulation of mitochondrial respiration by sperm-triggered Ca^{2+} oscillations has been observed (Schomer & Epel 1998, Dumollard *et al*. 2003, 2004, Campbell & Swann 2006). Thus, the lack of an oxidative burst observed in parthenotes could also be related to the single Ca^{2+} peak induced by parthenogenetic activation, which would not be efficient to stimulate mitochondria consistently.

There is scarce information about metabolic changes that occur in early bovine zygotes, especially in parthenogenetically activated oocytes. In the present work, we found clear and distinctive metabolic patterns between non-activated oocytes, IVF and parthenogenetically activated oocytes. Characteristic behaviors in redox activity and fluctuations of ROS production during early development could be integrated in our understanding of measurements of oocyte and early embryo competence. The differences observed in parthenogenetic zygotes with respect to these oxidative patterns could in part explain their impaired developmental competence. Further studies into the metabolic control of parthenogenetic activation could contribute to improve the performance of these embryos for different biotechnological applications, such as somatic cell nuclear transfer for genetic improvement through cloning and transgenesis.

Materials and Methods

The materials used in these experiments were obtained from Sigma–Aldrich, unless otherwise indicated.

Recovery and classification of COCs

Bovine ovaries were obtained from an abattoir within 30 min after killing and kept warm (30–33 °C) until they were brought to the laboratory. Ovaries were washed in physiological saline containing 100 000 IU/l penicillin and 100 mg/l streptomycin. COCs were recovered by aspiration of antral follicles (2–5 mm in diameter) and classified according to cumulus morphology under a stereomicroscope. Only oocytes completely surrounded by compact and multiple layers of cumulus cells were employed.

Oocyte IVM

Groups of 50 COCs were cultured in 500 µl medium 199 (Gibco) supplemented with 0.2 mg/l porcine FSH (Folltropin-V; Bioniche, Belleville, ON, Canada), 2 mg/l porcine LH (Lutropin-V; Bioniche), 5% (v/v) fetal bovine serum (Internegocios, Mercedes, Buenos Aires, Argentina), and 50 mg/l gentamicin sulfate under mineral oil (Squibb & Sons, Inc., Princeton, NJ, USA) at 39 °C for 22 h in an atmosphere of 5% CO_2 in humidified air.

Sperm-mediated activation of matured oocytes

IVF was carried out using frozen–thawed Holstein bull semen from a male of proven fertility. Semen was thawed at 37 °C in modified synthetic oviductal fluid (mSOF; Takahashi & First 1992) with 10 mmol/l theophylline, centrifuged at 500 ***g*** twice for 5 min, and then resuspended in fertilization medium to a final concentration of 2×10^6 motile spermatozoa/ml.

Co-incubation of COCs and spermatozoa was performed in IVF-mSOF medium, consisting of mSOF supplemented with 10 IU/ml heparin and 5 mg/ml BSA, under mineral oil at 39 °C in a humidified atmosphere of 5% CO_2 during 24 h. Different nuclear early embryo development stages were evaluated within 24 h of culture by the fluorescent stain Hoechst 33342 as described below.

Parthenogenetic activation of matured oocytes

Oocytes matured *in vitro* were denuded in PBS supplemented with 3 mg/ml BSA by gentle pipetting with a Pasteur pipette. Oocytes were considered mature when the first polar body was present.

Only mature oocytes were incubated in TALP supplemented with 3 mg/ml BSA with 5 μM ionomycin for 5 min and then in mSOF supplemented with 2 mM 6-dimethylaminopurine + 7.5 μg/ml cytochalasin B for 3 h as described by Grupen *et al.* (2002). They were then washed and placed in IVF-mSOF under mineral oil at 90% N_2:5% CO_2:5% O_2 and 100% humidity for 21 h.

Determination of redox state and nuclear stage

To determine redox state and nuclear stage, matured oocytes, putative zygotes, and parthenotes were collected from culture media at 2-h interval from 7 to 24 h post-activation/insemination. They were then denuded and incubated in PBS supplemented with 3 mg/ml BSA in the presence of 1 nM RedoxSensor Red CC-1 (Molecular Probes, Eugene, OR, USA) plus 1 μM Hoechst 33342 for 10 min in the dark at 39.5 °C.

All oocytes were then washed in PBS supplemented with 3 mg/ml BSA and mounted on glass slides. Fluorescence was measured by means of digital microphotographs using a Jenamed II epifluorescence microscope with an ×12 objective using 450–490 nm (excitation) and 570 nm (emission) filters for RedoxSensor Red CC-1. Pixel intensity within microphotographs of each oocyte/zygote/parthenote was determined using Image J 1.240 Software (National Institutes of Health, Federal Government of the United States). To normalize measurements between different replicates, the fluorescence of matured oocytes was set at a consistent level. Nuclear stage was evaluated at ×400 using 330–380 nm (excitation) and 420 nm (emission) filters for Hoechst 33342.

Redox state measurements were expressed as arbitrary units/oocyte or zygote/parthenote.

Determination of ROS production and nuclear stage

To measure ROS production and nuclear stage, matured oocytes, putative zygotes, and parthenotes were collected from culture media at 2-h intervals from 7 to 24 h post-activation/insemination, denuded, and incubated in PBS supplemented with 3 mg/ml BSA for 30 min in the presence of 5 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCHFDA; LeBel *et al.* 1992) and 1 μM Hoechst 33342. To measure esterase activity, 25% of the cells of each sample were incubated in the dark at 39.5 °C in PBS supplemented with 3 mg/ml BSA for 15 min in the presence of 0.12 μM fluorescein diacetate (FDA).

After exposure to DCHFDA plus Hoechst 33342 or FDA, all oocytes were washed in PBS supplemented with 3 mg/ml BSA and mounted on glass slides. Fluorescence was measured as described earlier using 450–490 nM (excitation) and 520 nM (emission) filters for DCHFDA and FDA.

Both DCHFDA and FDA fluorescence are dependent on the endogenous esterase activity, therefore, a pixel intensity ratio between DCHFDA fluorescence and the mean FDA fluorescence (for the subset measured) at each time point for each oocyte was determined as described by Lane *et al.* (2002). ROS levels were expressed as arbitrary ROS units/oocyte or zygote/parthenote.

In order to demonstrate the specificity of the dyes, COCs were cultured in medium 199 for 18 h, then denuded, and divided into two groups. One group of oocytes was incubated in medium 199 supplemented with 5 mM pyruvate for 2 h, while the rest remained in control medium for the same period. Pyruvate was chosen for the experiment because it has been described as an effective physiological antioxidant for its ability to scavenge hydrogen peroxide (Upreti *et al.* 1998).

Experimental design and statistical analysis

Data were expressed as mean ± S.E.M. Values at different time points were compared using ANOVA. A P value <0.05 was considered significant.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

Baker M & Aitken RJ 2004 The importance of redox regulated pathways in sperm cell biology. *Molecular and Cellular Endocrinology* **216** 47–54. (doi:10.1016/j.mce.2003.10.068)

Balaban RS, Nemoto S & Finkel T 2005 Mitochondria, oxidants, and aging. *Cell* **120** 483–495. (doi:10.1016/j.cell.2005.02.001)

Blondin P, Coenen K & Sirard M 1997 The impact of reactive oxygen species on bovine sperm fertilizing ability and oocyte maturation. *Journal of Andrology* **18** 454–460. (doi:10.1002/j.1939-4640.1997.tb01952.x)

Boveris A & Cadenas E 1975 Mitochondrial production of superoxide anions and its relationship to antimycin insensitive respiration. *FEBS Letters* **54** 311–314. (doi:10.1016/0014-5793(75)80928-8)

Boveris A & Cadenas E 1982 Production of superoxide radicals and hydrogen peroxide in mitochondria. In *Superoxide Dismutase*, pp 15–30. Ed. LW Oberley. Boca Raton: CRC Press.

Campbell K & Swann K 2006 Ca^{2+} oscillations stimulate an ATP increase during fertilization of mouse eggs. *Developmental Biology* **298** 225–233. (doi:10.1016/j.ydbio.2006.06.032)

Cetica PD, Pintos LN, Dalvit GC & Beconi MT 2001 Antioxidant enzyme activity and oxidative stress in bovine oocyte *in vitro* maturation. *IUBMB Life* **51** 57–64. (doi:10.1080/15216540152035073)

Chen CS & Gee KR 2000 Redox-dependent trafficking of 2,3,4,5,6-pentafluorodihydrotetramethylrosamine, a novel fluorogenic indicator of cellular oxidative activity. *Free Radical Biology & Medicine* **28** 1256–1278. (doi:10.1016/S0891-5849(00)00265-3)

Dalvit GC, Cetica PD, Pintos LN & Beconi MT 2005*a* Reactive oxygen species in bovine embryo *in vitro* production. *Biocell* **29** 209–212.

Dalvit GC, Llanes SP, Descalzo A, Insani M, Beconi M & Cetica P 2005*b* Effect of α-tocopherol and ascorbic acid on bovine oocyte *in vitro* maturation. *Reproduction in Domestic Animals* **40** 93–97. (doi:10.1111/j.1439-0531.2004.00522.x)

Dickinson DA & Forman HJ 2002 Glutathione in defense and signalling: lessons from a small thiol. *Annals of the New York Academy of Sciences* **973** 488–504. (doi:10.1111/j.1749-6632.2002.tb04690.x)

Downs SM & Mastropolo AM 1994 The participation of energy substrates in the control of meiotic maturation in murine oocytes. *Developmental Biology* **162** 154–168. (doi:10.1006/dbio.1994.1075)

Dumollard R, Hammar K, Porterfield M, Smith PJ, Cibert C, Rouviere C & Sardet C 2003 Mitochondrial respiration and Ca^{2+} waves are linked during fertilization and meiosis completion. *Development* **130** 683–692. (doi:10.1242/dev.00296)

Dumollard R, Marangos P, Fitzharris G, Swann K, Duchen M & Carrol J 2004 Sperm-triggered [Ca^{2+}] oscillations and Ca^{2+} homeostasis in the mouse egg have an absolute requirement for mitochondrial ATP production. *Development* **131** 3057–3067. (doi:10.1242/dev.01181)

Dumollard R, Ward Z, Carroll J & Duchen R 2007 Regulation of redox metabolism in the mouse oocyte and embryo. *Development* **134** 455–465. (doi:10.1242/dev.02744)

Funato Y, Micchiue T, Asashima M & Miki H 2006 The thioredoxin-related redox-regulating protein nucleoredoxin inhibits Wnt–β-catenin signalling through disheveled. *Nature Cell Biology* **8** 501–508. (doi:10.1038/ncb1405)

Gordon I 1994 *In vitro* fertilization. In *Laboratory Production of Cattle Embryos*, 1st edition, ch 4, pp 170–226. Ed I Gordon. Wallingford, UK: CAB International.

Grupen CG, Mau JC, Mc Ilfatrick SM, Maddocks S & Nottle MB 2002 Effect of 6-dimethylaminopurine on electrically activated *in vitro* matured porcine oocytes. *Molecular Reproduction and Development* **62** 387–396. (doi:10.1002/mrd.10126)

Han Z, Vassena R, Chi MMY, Potireddy S, Sutovsky M, Moley KH, Sutovsky P & Latham KE 2008 Role of glucose in cloned mouse embryo development. *American Journal of Physiology. Endocrinology and Metabolism* **295** E798–E809. (doi:10.1152/ajpendo.00683.2007)

Hancock JT, Desikan R & Neill SJ 2001 Role of reactive oxygen species in cell signalling pathways. *Biochemical Society Transactions* **29** 345–360. (doi:10.1042/BST0290345)

Hashimoto S, Minami N, Yamada M & Imai H 2000 Excessive concentration of glucose during *in vitro* maturation impairs the developmental competence of bovine oocytes after *in vitro* fertilization: relevance to intracellular reactive oxygen species and glutathione contents. *Molecular Reproduction and Development* **56** 520–526. (doi:10.1002/1098-2795(200008)56:4<520::AID-MRD10>3.0.CO;2-0)

Imai S, Johnson FB, Marciniak RA, Mc Vey M, Park PU & Guarente L 2000 Sir2: an NAD-dependent histone deacetylase that connects chromatin silencing, metabolism, and aging. *Cold Spring Harbor Symposia on Quantitative Biology* **65** 297–302. (doi:10.1101/sqb.2000.65.297)

Jiang HS 1991 Studies in the culture of bovine embryos derived from oocytes matured and fertilized *in vitro*. *PhD Thesis*. National University of Ireland, Dublin.

Kim JH, Niwa K, Lim JM & Okuda K 1993 Effects of phosphate, energy substrates, and amino acids on development of *in vitro*-matured, *in vitro*-fertilized bovine oocytes in a chemically defined, protein-free culture medium. *Biology of Reproduction* **48** 1320–1325. (doi:10.1095/biolreprod48.6.1320)

Kriaucionis S & Heintz N 2009 The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. *Science* **324** 929–930. (doi:10.1126/science.1169786)

Lane L, Maybach JM & Gardner DK 2002 Addition of ascorbate during cryopreservation stimulates subsequent embryo development. *Human Reproduction* **17** 2686–2693. (doi:10.1093/humrep/17.10.2686)

LeBel C, Ischiropoulos H & Bondy S 1992 Evaluation of the probe 2′,7′-dichlorofluorescein as an indicator of reactive oxygen species formation and oxidative stress. *Chemical Research in Toxicology* **5** 227–231. (doi:10.1021/tx00026a012)

Lim KT, Jang G, Ko KH, Lee WW, Park HJ, Kim JJ, Lee SH, Hwang WS, Lee BC & Kang SK 2007 Improved *in vitro* bovine embryo development and increased efficiency in producing viable calves using defined media. *Theriogenology* **67** 293–302. (doi:10.1016/j.theriogenology.2006.07.011)

Liu H, Colavitti R, Rovira II & Finkel T 2005 Redox dependent transcriptional regulation. *Circulation Research* **97** 967–974. (doi:10.1161/01.RES.0000188210.72062.10)

Lopes AS, Lane M & Thompson JG 2010 Oxygen consumption and ROS production are increased at the time of fertilization and cell cleavage in bovine zygotes. *Human Reproduction* **25** 2762–2773. (doi:10.1093/humrep/deq221)

Monaghan P 1993 Studies related to the laboratory production of bovine embryos. *PhD Thesis*. National University of Ireland, Dublin.

Morado S, Cetica P, Beconi M & Dalvit G 2009 Reactive oxygen species in bovine oocyte *in vitro* maturation. *Reproduction, Fertility, and Development* **21** 608–614. (doi:10.1071/RD08198)

Nakamura Y, Yamagata Y, Sugino N, Takayama H & Kato H 2002 Nitric oxide inhibits oocyte meiotic maturation. *Biology of Reproduction* **67** 1588–1592. (doi:10.1095/biolreprod.102.005264)

Nasr-Esfahani M & Johnson M 1991 The origin of reactive oxygen species in mouse embryos cultured *in vitro*. *Development* **113** 551–560.

Rahman I, Marwick J & Kirkham P 2004 Redox modulation of chromatin remodeling: impact on histone acetylation and deacetylation, NF-κB and pro-inflammatory gene expression. *Biochemical Pharmacology* **68** 1255–1267. (doi:10.1016/j.bcp.2004.05.042)

Rivlin J, Mendel J, Rubinstein S, Etkovitz N & Breitbart H 2004 Role of hydrogen peroxide in sperm capacitation and acrosome reaction. *Biology of Reproduction* **70** 518–522. (doi:10.1095/biolreprod.103.020487)

Ruggeri RR, Watanabe Y, Meirelles F, Bressan FF, Frantz N & Bos-Mikich A 2012 The use of parthenotegenetic and IVF bovine blastocysts as a model for the creation of human embryonic stem cells under defined conditions. *Journal of Assisted Reproduction and Genetics* **29** 1039–1043. (doi:10.1007/s10815-012-9866-z)

Schomer B & Epel D 1998 Redox changes during fertilization and maturation of marine invertebrate eggs. *Developmental Biology* **203** 1–11. (doi:10.1006/dbio.1998.9044)

Shirazi A, Nazari H, Ahmadi E, Heidari B & Shams-Esfandabadi N 2009 Effect of culture system on survival rate of vitrified bovine embryos produced *in vitro*. *Cryobiology* **59** 285–290. (doi:10.1016/j.cryobiol.2009.08.003)

Sikka SC 2001 Relative impact of oxidative stress on male reproductive function. *Current Medicinal Chemistry* **8** 851–862. (doi:10.2174/0929867013373039)

Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, Agarwal S, Iyer LM, Liu DR, Aravind L *et al.* 2009 Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science* **324** 930–935. (doi:10.1126/science.1170116)

Takahashi Y & First NL 1992 *In vitro* development of bovine one-cell embryos: influence of glucose, lactate, pyruvate, amino acids and vitamins. *Theriogenology* **37** 963–978. (doi:10.1016/0093-691X(92)90096-A)

Takami M, Preston SL, Toyloy VA & Behrman HR 1999 Antioxidants reversibly inhibit the spontaneous resumption of meiosis. *American Journal of Physiology* **276** 684–688.

Tatemoto H, Ootaki K, Shigeta K & Muto N 2001 Enhancement of developmental competence after *in vitro* fertilization of porcine oocytes after treatment with ascorbic acid 2-*O*-α glucoside during *in vitro* maturation. *Biology of Reproduction* **65** 1800–1806. (doi:10.1095/biolreprod65.6.1800)

Thompson JG, Partridge RJ, Houghton FD, Cox CI & Leese HJ 1996 Oxygen uptake and carbohydrate metabolism by *in vitro* derived bovine embryos. *Journal of Reproduction and Fertility* **106** 299–306. (doi:10.1530/jrf.0.1060299)

Upreti GC, Jensen K, Munday R, Duganzich DM, Vishwanath R & Smith JF 1998 Studies on aromatic amino acid oxidase activity in rat spermatozoa: role of pyruvate as an antioxidant. *Animal Reproduction Science* **51** 275–287. (doi:10.1016/S0378-4320(98)00082-7)

Wells D, Micika P & Tervit H 1999 Production of clone calves following nuclear transfer with cultured adult mural granulose cells. *Biology of Reproduction* **60** 996–1005. (doi:10.1095/biolreprod60.4.996)

Wossidlo M, Nakamura T, Lepikhov K, Marques CK, Zakhartchenko V, Boiani M, Arand J, Nakano T, Reik W & Walter J 2011 5-Hydroxymethylcytosine in the mammalian zygote is linked with epi genetic reprogramming. *Nature Communications* **2** 241. (doi:10.1038/ncomms1240)

Yang HW, Hwang KJ, Kwon HC, Kim HS, Choi KW & Oh KS 1998 Detection of reactive oxygen species (ROS) and apoptosis in human fragmentated embryos. *Human Reproduction* **13** 998–1002. (doi:10.1093/humrep/13.4.998)

Zhang Q, Piston DW & Goodman RH 2002 Regulation of corepressor function by nuclear NADH. *Science* **295** 1895–1897. (doi:10.1126/science.1069300)

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